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COMPARATIVE STUDY OF THE EFFECTS OF SALTS ON FOUR ENZYMES FROM THE EXTREME HALOPHILE BACTERIA OF HALOBACTERIUM CUTIRUBRUM

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16. Abstract

The effects of monovalent and bivalent cations, as chlorides, on three dehydrogenases and one synthetase partially purified from H.cutirubrum were studied. All monovalent cations tested were effective , with the exception of TRIS for glycerol dehydrogenase. The effectiveness of the same cations differed for the different enzymes. All enzymes were activated by low concentrations of Ca^{++} or Mg^{++}. The enzymes were rapidly inactivated when incubated at 30° with low salt concentration; they were protected with varying effectiveness by the same salts tested as activators. No correlation was observed in the effectiveness of the salts as activator and as stabilizers of the four enzymes, or as activators and protectors of a same enzyme. The order of effectiveness of the anions Cl^-, Br^-, NO_3 and SCN^-, as K^+ salts, as activators of the enzymes studied followed their effectiveness in salting out.

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COMPARATIVE STUDY OF THE EFFECTS OF SALTS ON FOUR ENZYMES FROM THE EXTREME HALOPHILE BACTERIA OF HALOBACTERIUM CUTIRUBRUM

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Introduction

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In order to grow, extreme halophile bacteria require a NaCl concentration close to saturation, and they exhibit extremely high intracellular saline concentrations; their enzymes also require high concentrations of salt in order to be both active and stable [1, 3]. The two roles proposed for the salts, as activators and stabilizers, namely the shielding of negative charges in the protein molecule, suggested by Baxter [4], and the reinforcement of the hydrophobic links of the protein, suggested by Lanyi [5], do not actually exclude one another and probably complement one another [6]. Although the properties of different halophile enzymes have been studied in the last decade [2, 3], the results are perhaps too limited to permit generalizations; for example, Lanyi's hypothesis was developed on the basis of three cases studied with raw extracts, two of them corresponding to particulate enzyme systems [5, 7].

The purpose of this study is to compare the effectiveness of different salts as activators and stabilizers for four partially purified enzymes from Halobacterium cutirubrum, namely 3 dehydrogenases, glycerol dehydrogenase (EC 1.1.1.6), glutamate dehydrogenase (EC 1.4.1.3) and malate dehydrogenase (EC 1.1.1.37), and one synthetase, citrate synthetase (EC 4.1.3.7).

* Numbers in the margin indicate pagination in the foreign text.

Materials and Methods

Organism. The Halobacterium cutirubrum strain was kindly supplied by Dr. D. J. Kushner, of the Faculty of Science and Engineering, University of Ottawa, Canada.

Culture. The microorganism was developed in a rich medium [8] (1.5 ℓ) at 38°. The cultures were stirred and aerated by causing air bubbles to pass through them with the aid of a vacuum pump. The cells were harvested when the cultures attained an absorbancy of about 0.7 at 680 nm (usually after 4 to 5 days, starting with an initial absorbancy of 0.05). The yield was approximately 3.3 g (moist weight) per liter of medium.

Obtaining enzyme preparations. The cells were harvested by centrifugation at 10,000 × g during 15 min at 4°; they were washed in 0.05 M TRIS-HCl buffer (pH 7.6) containing 5 M of Na Cl and 1 mM of EDTA, and they were suspended in 0.45 ml of the same buffer per gram (moist weight).

The cells were broken up by ultrasonic waves (four treatments with a duration of 15 sec each, at 5°) in an MSE (Measuring and Scientific Equipment, London) ultrasonic generator operating at full power. $MgCl_2$ (up to a final concentration of 2 mM) and DNA-ase were then added to the homogenate in a proportion of 20 µg/ml of suspension. After an incubation of 30 min at 30°, the homogenate was centrifugated at 37,000 × g for 45 min at 4°. The slightly turbid, dark red supernatant was retained as a raw extract, and the precipitate was discarded.

The enzymes studied were partially purified by fractionation of the raw extract in 0.05 M of a TRIS-HCl buffer (pH 7.6) containing 5 M of NaCl and 1 mM of EDTA with a saturated solution of $(NH_4)_2SO_4$ (containing 1 mM of EDTA adjusted to pH 7 with NH_4OH).

Wtl

While it was not possible to effectively precipitate the halophile proteins with solid $(NH_4)_2SO_4$ in the presence of 5 M of NaCl, said fractionation was possible with a saturated solution, due to the dilution of the NaCl that occurred simultaneously with the addition of $(NH_4)_2SO_4$.

The raw extract was taken successively to $(NH_4)_2SO_4$ concentrations corresponding to 66, 77.6, 83.3, 92, 94.3 and 100% saturation at 0°. In every case the addition of the saturated solution (or sodium salt for the 100% saturation fraction) was carried out slowly and with proper stirring; following an additional period of 10 min of stirring, the suspensions were centrifugated at 37,000 × g for 20 min. The substantial precipitates obtained in the first two fractionations, which contained about 60% of the total protein in the raw extract, including the major portion of the particulate material, were washed in solutions containing the corresponding concentrations of NaCl and $(NH_4)_2SO_4$, and the liquids resulting from the wash were mixed with the corresponding supernatants.

The glycerol dehydrogenase [GDH] was purified about ten times with a 50% yield from the 83.3% saturation fraction; the glutamate dehydrogenase [GluDH], the malate dehydrogenase [MDH] and the citrate synthetase [CS] were purified about 13 times with yields of the order of 25% of the 100% saturation fraction. The corresponding precipitates of these fractions were redissolved in the TRIS-HCl-NaCl-EDTA buffer, dialyzed against the same solution for the purpose of eliminating the remaining $(NH_4)_2SO_4$, and used for the experiments described.

Measurement of enzyme activity. All the enzymes studied were determined in a Beckman DB-G recording spectrophotometer, thermostated at 30°, at 340 nm for the dehydrogenases, or at 412 nm for the citrate synthetase. In every case, the reactions

were started with the addition of the enzyme to the reaction mixture, in order to minimize the problems caused by enzyme denaturalization in the presence of low saline concentrations. All of the salt solutions contained the buffer consisting of 50 mM of TRIS-HCl and 1 mM of EDTA, and were adjusted to pH 7.6 after the addition of salt.

The reaction mixtures contained, in a final volume of 1 ml, /294 the following quantities of reactants (in micromoles):

GDH: TRIS-HCl, 31; dehydroxyacetone, 2; nicotinamide adenine denucleotide, reduced [NADH], 015.

GluDH: TRIS-HCl, 11, α-KG, 2; nicotinamide adenine denucleotide phosphate, reduced [NADPH], 015; $ClNH_4$, 80.

MDH: TRIS-HCl, 16; oxalacetate [OAA], 0.5; NADH, 0.15;

CS: TRIS-HCl, 40; OAA, 1, acetyl-coenzyme A, [CoA], 0.15; 5,5'-dithiobis(2-nitrobenzoic) acid [DTNB], 0.1.

The quantities of salts and enzymes were indicated in the table captions.

The stabilization experiments were conducted as previously described for the halophile malic enzyme [1, 9]. The half-life values in minutes obtained from the semilogarithmic graphs of the remaining activity versus the time of incubation at 30° are shown in Table 5, as a measure of the stabilizing effects of the salts.

Protein determination. The protein was determined by means of the method of Lowry and col. [10]. No interference was caused by the TRIS or the salts with the quantities of enzyme preparations used (not greater than 0.01 ml).

Reagents. The NADH, CoA and OAA were obtained from Boehringer, Mannheim, Germany; the NADPH, dihydroxyacetone, DNA-ase I (of steer pancreas), DTNB, TRIS, and β-mercaptoethanol,

from Sigma Chemical Company, St. Louis, Mo., USA; α-KG (disodium salt), from Th. Schuchardt, Munich, Germany. All the salts used were analytical reagents from Merck, Darmstadt, Germany or from the British Drug Houses, Poole, England. The acetyl-CoA was prepared from CoA and acetic anhydride by the Stadtmann method [11].

Results and Discussion

Tables 1, 2, 3, and 4 show the activation of GDH, GluDH, MDH and CS, respectively, with various concentrations of (1) monovalent-cation chlorides; (2) bivalent-cation chlorides; and (3) K^+ salts with different anions. In the case of GluDH, where NH_4^+ is a reaction substrate, a fixed concentration of NH_4^+ (80 mM) was used in all determinations, in addition to the variable concentrations of the salts studied. This concentration, corresponding to double the apparent K_m for $ClNH_4$ as a substrate (in the presence of 0.35 M of LiCl), was incapable of activating or protecting the enzyme by itself. Since it was not a saturating concentration, the activity values in the presence of increasing concentrations of NH_4Cl as the activator were not taken into account account for the calculation of 100% activation, although they are included in Table 2.

The monovalent cation chlorides tested were capable of activating all of the enzymes studied, with the sole exception of TRIS-HCl in the case of GDH (Table 1). The bivalent cations tested, Ca^{++} and Mg^{++}, were not only effective as activators in every case, but Ca^{++} was the best activator, superior to the monovalent cations in the cases of GluDH and MDH. The activator effect of the Mg^{++} on the halophile NADH oxidase [12] and cytochrome oxidase [7] was previously described, but in both cases the effect was considerably less than in the case of the monovalent cations, Na^+ and K^+.

TABLE 1. ACTIVATION OF HALOPHILE GLYCEROL DEHYDROGENASE BY SALTS

The enzyme activity, determined in the presence of saline concentrations indicated in this table, as described in materials and methods, is expressed as a percentage of the maximum activity achieved in the experiment (33 nmoles min, in the presence of 2-3 M NH_4Cl). 79 µg of protein were used per determination.

Conc. (M)	NaCl	LiCl	TRIS HCl	NH_4Cl	KCl	KBr	KNO_3	KSCN	$MgCl_2$	$CaCl_2$
0	0	0	0	0	0	0	0	0	0	0
0.005									45	44
0.010									46	45
0.020				41	23				44	46
0.050				69	28				35	44
0.100	2	14	0	73	35		27	22	31	39
0.200	8	27	0	77	44	36	34	25	21	37
0.400	33	27	1	63	48	36	30	20	8	24
1.000	29	22	2	92	53	30	25	8		
2.000	26	14	1	100	64	26	20	1		
3.000	27	11	0	100	77	25		0		

The effectiveness of the cations as activators depended not /297
only on the nature of the cation, but also on the nature of the anion, as shown by the comparison of the effects of four K^+ (Cl^-, Br^-, NO_3^- and SCN^-) salts (Tables 1 to 4). The SCN^- was a poor activator in every case, and the order of effectiveness of the anions, the same as in the case of the cations, was Cl^- > Br^- >> > NO_3^- > SCN^-. This is the same order of effectiveness of the anions as agents capable of causing salting out, i.e., of reducing the solubility of different substances in water [13], and it would be related to the effects of the salt on "halophile" hydrophobic combinations, in the sense that they would not be stabilized only by the presence of water, since they would also require the presence of salts such as the NaCl in high concentrations [5]. The inhibition by KSCN, however, cannot be considered

TABLE 2. ACTIVATION OF HALOPHILE GLUTAMATE DEHYDROGENASE BY SALTS

The enzyme activity, determined in the presence of saline concentrations indicated in this table, and described in materials and methods, is expressed as a percentage of the maximum activity achieved in the experiment (14 nmoles/min, in the presence of 0.2 M $CaCl_2$). Activation by NH_2Cl is not considered for the purposes of calculation of 100% activity, since the concentration of NH_4Cl as a substrate used in the presence of other cations (80 mM) was not saturating. 63 µg of protein were used per determination.

Conc. (M)	NaCl	LiCl	TRIS-HCl	NH_4Cl	HCl	KBr	KNO_3	KSCN	$MgCl_2$	$CaCl_2$
0	0	0	0	0	0	0	0	0	0	0
0.005									45	36
0.010									47	47
0.020									58	52
0.050									81	69
0.100	36	16	25	0	15				97	93
0.200	59	49	48	36	37	50	10	2	92	100
0.400	81	73	67	95	77	51	10	1	56	81
1.000	77	95	66	121	76	23	5	0		
2.000	41	65	23	66	62	15	2	0		
3.000	32	34	10	31	47	7	0			

as a necessary indication of the presence of such "halophile" hydrophobic combinations, since the KSCN is a salt capable of producing salting in, i.e., of favoring the aqueous solution of slighly soluble substances [13], and, consequently, it might destroy "normal" hydrophobic combinations, which usually participate in maintaining the tertiary structure of proteins. The effectiveness of the anions, in the order mentioned, for the activation of menadione reductase [5] and of cytochrome oxidase [7] was previously described by Lanyi and collaborators.

The four enzymes studied were inactivated by dialysis from the 0.05 TRIS-HCl buffer (pH 7.6) containing 1 mM of EDTA and

TABLE 3. ACTIVATION OF HALOPHILE MALATE DEHYDROGENASE BY SALTS

The enzyme activity, determined in the presence of saline concentrations indicated in this table, and described in materials and methods, is expressed as a percentage of the maximum activity achieved in the experiment (41 nmoles/min, in the presence of $CaCl_2$, 0.4 M). 21 µg of protein were used per determination.

Conc.(M)	NaCl	LiCl	TRIS-HCl	NH_4Cl	KCl	KBr	KNO_3	KSCN	$MgCl_2$	$CaCl_2$
0	8	8	8	8	8	8	8	8	8	8
0.005									21	38
0.010									31	49
0.020									41	64
0.050									51	78
0.100	18	18	26	18	21	30	33	31	61	92
0.200	23	24	30	27	28	40	40	37	66	98
0.400	33	36	41	42	42	52	49	36	46	100
1.000	60	61	57	66	66	68	55	17		
2.000	51	51	46	54	55	57	52	0		
3.000	36	34	26	41	41	38		0		

TABLE 4. ACTIVATION OF HALOPHILE CITRATE SYNTHETASE BY SALTS

The enzyme activity, determined in the presence of the saline concentrations indicated in this table, and described in materials and methods, is expressed as a percentage of the maximum activity achieved in the experiment (11 nmoles/min, in the presence of KCl, 3 M). 10 µg of protein were used per determination.

Conc.(M)	NaCl	LiCl	TRIS-HCl	NH_4Cl	KCl	KBr	KNO_3	KSCN	$MgCl_2$	$CaCl_2$
0	9	9	9	9	9	9	9	9	9	9
0.005									42	49
0.010									55	66
0.020									64	72
0.050									71	87
0.100	34	35	27	28	38	41	26	16	46	19
0.200	50	48	37	41	56	50	34	5	30	45
0.400	64	56	44	51	66	53	36	0	16	24
1.000	63	51	41	44	73	41	8	0		
2.000	63	35	35	24	78	23	3	0		
3.000	64	29	29	6	100	12		0		

2 mM of β-mercaptoethanol, for 3 hours at 4°. The activities of MDH and CS could be partially recovered (62 and 78%, respectively) by dialysis from the 0.05 M TRIS-HCl buffer (pH 7.6) containing 5 M of NaCl and 1 mM of EDTA during 3 hours at 23°; the activities of GDH and GluDH were not recovered under these conditions.

This inactivation of the enzymes studied could have been prevented with a different degree of effectiveness by the same mono- and bivalent cation salts tested as activators (Table 5). The concentrations used were 1 M for the monovalent cations, and 0.1 M for the bivalent cations; the reason for this choice was that these values gave good activations, which were close to optimum, in the majority of cases (Tables 1 to 4). The temperature used to determine protection by means of the salt was the same as the one used to determine activation (30°) in order to permit a direct comparison of the effectiveness of the salts in both processes.

TABLE 5. STABILIZATION OF HALOPHILE ENZYMES BY SALTS

The stability of enzymes at 30° was determined as described in materials and methods. The concentrations of protein (μg/ml) and the basic concentration of NaCl (M) in the preincubation mixtures were, respectively: GDH, 158 and 0.1; GluDH, 52 and 0.1; MDH, 54 and 0.1; CS, 20 and 0.2. The half-life values are expressed in min.

Salt	Without addition	NaCl	LiCl	TRIS-HCl	NH_4Cl	KCl	$MgCl_2$	$CaCl_2$
Concentration (M)	Basal	1	1	1	1	1	0,1	0,1
GDH	0,1	960	300	0,3	1860	3240	2460	1140
GluDH	0,1	5400	6	12	140	132	9	8
MDH	0,1	450	22	72	108	75	220	360
CS	0,1	145	8	1,5	2,5	53	0,2	0,5

TABLE 6. COMPARISON OF THE ORDER OF EFFECTIVENESS OF CATIONS (IN THE FORM OF CHLORIDES) AS ACTIVATORS AND STABILIZERS OF HALOPHILE ENZYMES

The order of effectiveness shown is a qualitative summary of the data contained in Tables 1 to 5, considering the maximum activation obtained with each cation. In the case of GluDH, NH_4Cl was not taken into account.

Enzyme		Order of effectiveness
GDH	Activation	$NH_4^+ > K^+ > Mg^{++} = Ca^{++} > Na^+ > Li^+ >> Tris$
	Stabilization	$K^+ > Mg^{++} > NH_4^+ > Ca^{++} > Na^+ > Li^+ >> Tris$
GluDH	Activation	$Ca^{++} = Mg^{++} > Li^+ > Na^+ > K^+ > Tris$
	Stabilization	$Na^+ >> K^+ = NH_4^+ >> Tris > Mg^{++} \cong Ca^{++} \cong Li^+$
MDH	Activation	$Ca^{++} > K^+ = NH_4^+ = Mg^{++} > Na^+ = Li^+ = Tris$
	Stabilization	$Na^+ > Ca^{++} > Mg^{++} > NH_4^+ > K^+ = Tris > Li^+$
CS	Activation	$K^+ > Ca^{++} > Mg^{++} > Na^+ > Li^+ > NH_4^+ > Tris$
	Stabilization	$Na^+ > K^+ >> Li^+ > NH_4^+ > Tris > Ca^{++} > Mg^{++}$

The results in Tables 1 to 5 are qualitatively summarized in Table 6, in order to facilitate the comparison of the effectiveness of the salts as activators and stabilizers of the different enzymes studied, and as activators and stabilizers of each of the enzymes. We can see that cations with a similar hydrated ion radius, such as K^+ and NH_4^+, were similar as activators of GDH and MDH, but behaved quite differently in the case of CS. GDH showed a marked preference for K^+ and NH_4^+ as activators. In the case of MDH, all cations were effective to a similar degree /298 throughout the entire concentration range studied; however, K^+ and NH_4^+ were slightly superior as activators to Na^+ and Li^+. The order of effectiveness of the cations as activators of GluDH and CS changed with the cation concentration, as previously proved by Aitken and coll. [14] in the case of the halophile isocitrate dehydrogenase. The order of effectiveness of the salts as stabilizers was also different for the different enzymes, though

NaCl was the best protector except in the case of GDH. When the order of effectiveness of the same cations as stabilizers and activators of one same enzyme was compared (Table 6), it was possible to see that both series were similar only in the case of GDH. Marked differences were observed in all other cases.

Similar studies conducted with a raw preparation of the dependent malic NADP enzyme (EC 1.1.1.40) [9] and with a partially purified preparation of aspartate amino transferase (EC 2.6.1.1) (A. I. Higa and J. J. Cazzulo, unpublished observations), isolated from the H. cutirubrum, also showed differences of the order of effectiveness of the salts as activators and stabilizers. These differences were particularly marked in the case of the malic enzyme, where NH_4^+ was the best activator but produced virtually no stabilization, and Na^+ was the best stabilizer but produced no activation whatsoever [9].

These results are difficult to explain if only Baxter's hypothesis [4] is taken into account, since if only the protection of the negative charges were important in the enzyme protein, a similar order of effectiveness of the salts both for activation and stabilization of the different halophile enzymes could be expected. However, the fact that the bivalent cations were more effective as activators than the monovalent cations, at least in terms of optimal concentration, suggests that the protection of charges, where the bivalent cations were more effective than the monovalent cations [5], played an important part in the activation of the enzymes studied. /299

The effect of a cation was substantially modified by the nature of the accompanying anion, in a manner compatible with Lanyi's hypothesis [5]. The behavior of the soluble enzymes studied here was not, however, entirely similar to that of the menadione reductase [5] and of the cytochrome oxidase [7]. The

latter were much better activated by monovalent cations than by bivalent cations; the only enzyme studied here that exhibited similar properties, GDH (Table 1), was not particularly sensitive to inhibition by kaotropic salts [13]. GluDH, on the contrary, which was substantially inhibited by the kaotropic salts (Table 2) was even better activated by Ca^{++} and Mg^{++} than by the mono-monovalent-cation salts tested. Both GluDH and CS, which were the most sensitive of the enzymes studied here with respect to inhibition by KNO_3 and KSCN, were hardly protected by the bivalent-cation salts, which were, nevertheless, efficient activators. If, following Lanyi's criterion [5, 7], we admit that these properties are directly related to the importance of the hydrophobic bonds in maintaining the enzyme structure, we might conclude that there are hydrophobic bonds of particular importance for the stability of the halophile GluDH and CS, but of lesser importance for activity. In the case of MDH, it does not seem likely that the "halophile" hydrophobic bonds suggested by Lanyi are principally responsible for maintaining the stable and active structure. In effect, (1) the enzyme was active to a maximum degree in the presence of $CaCl_2$, 0.1-0.4 M, which are concentrations supposed to be incapable of producing a marked salting out effect [7]; (2) the monovalent-cation salts produced a relative inhibition above 1 M, i.e., in the area of concentration where the protection of charges is complete [5], and above which salting out effects became manifest; (3) the $CaCl_2$ and the $MgCl_2$ in low concentration (0.1 M) were effective stabilizers of the enzyme. /300

If we consider the differences set forth, disclosed by the comparison of only four halophile enzymes under identical experimental conditions, it would appear to be premature at this time to attempt a unified explanation concerning the behavior of all halophile enzymes. Such an hypothesis should take into account not only the differences in the individual characteristics of the cations as activators and stabilizers, but also the

differences in the order of effectiveness of the salts as activators and stabilizers of one same enzyme, indicating different roles for the salts in both processes [9].

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